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# Notes on the blackening of Baptisia tinctoria

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Any one who is accustomed to the aspect of our dry open fields or sandy hills in the summer can not have failed to notice the Wild Indigo (Yellow Indigo Broom, *Baptisia tinctoria* (L.) R. Br.). The bright yellow flowers and small three-parted leaves make a very conspicuous plant. In early July, on Cape Cod, before the flowers are open the plants are green, but a few weeks later a black twig or branch will be noticed, and by August often whole plants will be black, even the flowers. If a green leaf is pinched or injured so that the epidermis is broken, there will presently be a black spot at the injury, and it is generally the branches which have been stepped on or have suffered from an insect burrowing into the stem near the ground, which are the first to show this very characteristic blackening. It is not always due to an injury; an apparently healthy plant will be so black that one can easily believe that a good substitute for indigo could be found in it, and indeed during the Civil War this was tried; while in drying it is well known how dark a specimen it makes.

The following facts were noted at the Marine Biological Laboratory at Woods Hole, Massachusetts, during some studies made at Dr. True's suggestion and with his kind assistance, upon the cause of the blackening of the leaves of *Baptisia tinctoria*. It was thought probably to be due to an oxidizing enzyme, and various experiments were tried to prove whether or not this was the case.

It may be well to say a word about the enzymes. Effront *

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* Effront, J. Les enzymes et leurs applications, 63, 64. 1899.

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has classified them into three large groups according to the effects which they produce:

A. Soluble hydrating ferments or enzymes.
B. Soluble oxidizing enzymes.
C. Enzymes causing molecular decomposition.

It is with the middle group that this paper is concerned. The oxidizing enzymes are found in both animals and plants, are widely distributed in all the parts of plants and in every kind of plant from bacteria to the phanerogams. Laccase, the first of the oxidases to be discovered (in 1883 by Yoshida, a Japanese chemist), makes lacquer; malase acts on fruits and causes apples and pears to turn brown when exposed to air; and others are connected with the ripening of olives or are found in wine. Without giving them actual names, like the foregoing, there are two kinds of oxidases generally recognized by the differences of their reactions, one "oxidase," which oxidizes guaiaconic acid (the characteristic reactive in the guaiac resin) to guaiac blue without the aid of peroxide of hydrogen, and the other "peroxidase," which oxidizes it only when this substance is present.*

There is a third, catalase, which according to Loew † is of general occurrence in the vegetable kingdom.

In this paper only very general experiments are described, but the methods of preparation of the material and ways of applying the tests are the ones usually adopted for this kind of work.

*Preparation of material.* — Fresh stems were gathered and the leaves stripped off; flowers also were used when present, but not the pod or thicker stems, as these could not easily be sufficiently broken up. The leaves were ground up in a mortar with quite fine sand, which had been previously treated with dilute hydrochloric acid and washed until no acid reaction could be detected with litmus paper, then dried in the sun. When ground, the leaves were covered with distilled water and left to macerate for a period of a half to one hour; the extract was next filtered, the leaves being squeezed in a cloth as dry as possible and thrown away. The filtrate was treated with three times its bulk of strong alcohol and left for an hour or two, to precipitate chlorophyl, proteins and other matters; this precipitate, dissolved in distilled water, was filtered again, and this solution used for the experiments.

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*Loew, O. Curing and fermentation of cigar leaf tobacco, 27. (U. S. Dept. Agriculture Rep. No. 59.) 1899.

† Loew, O. Catalase, 30. (U. S. Dept. Agriculture Rep. No. 68.) 1901.

*Kinds of extracts made.* — It was found that as soon as the pounded leaves were exposed to the air they turned from green to black in less than half an hour, whereas those not so much exposed kept green. So two kinds of extracts were made, one in which the pounded leaves were at once covered with water, when only those near the top became black, another in which the pounded leaves were left for twenty-four hours to get as black as possible in the air, and then extracted as usual. The first method gave a dark brown solution which kept growing darker so that it was difficult to see the color-reactions; the second gave a clear amber-colored extract, free from the products of oxidation, easily showing the slightest reaction to color tests, but otherwise acting like the first. In order to get purer enzyme solutions, a portion of the original solution was treated with three times its bulk of alcohol and filtered, dissolving the precipitate in water, and this process was repeated, thus securing second and third extractions much lighter and clearer and rather more sensitive than the first.

In general 95 per cent. alcohol was used for the first treatment, and 80 per cent. for the second and third. Although some absolute alcohol was also used for the first extraction and gave much heavier precipitates, it was too expensive to use in the necessary quantities. There was little difference in the amount of precipitate between 95 per cent. and 80 per cent. alcohol.

*Tests for enzymes.* — When the extracts were ready they were tested by taking about ten cubic centimeters in a tube and adding a few drops of solution of gum guaiac (2 grams of guaiac in 100 cubic centimeters of absolute alcohol); then the solution turned an opalescent blue, becoming slightly darker when shaken, and a clear blue on adding 95 per cent. alcohol, which dissolves the excess of guaiac. This showed that an oxidase was undoubtedly present. Upon the addition of one drop of hydrogen peroxide, the solution turned a very deep blue, and this color would last several hours without fading. This second reaction suggested the presence of a peroxidase. These two tests were used in all the experiments, the colors given above being considered the normal reaction and any variations, as the rapidity in the change, or shades of blue, were taken to mark disturbance in the action of the enzyme. It was found that the guaiac solution did not keep its power more than three or four days. The best reactions were produced the day it was made up. Green * considers that it is not safe to rely upon the guaiac test as a method of recognition for an oxidase, as several ordinary chemicals and mineral compounds also yield a blue color with it, and says the surer way is to isolate the enzyme and prove its presence by its action. On the other hand Loew † considers that "such objections are groundless when fresh organized tissues are under investigation. Other oxidizing influences are easily recognized by control tests, which the careful worker will not omit to make. . . . Such blue reactions are not restricted to certain limits of temperature as the reactions of oxidizing enzymes are."

*Temperature experiments.* — These were made by putting five or ten cubic centimeters of the enzyme solution into tubes and exposing them for five, ten, fifteen and thirty minutes as nearly as possible to the same temperature, by means of water baths. Exposures begun at 55° C. and up to 75° C. showed no difference from the normal. At 75° C. for shorter exposures the guaiac or oxidase reaction was slower in appearing and not such a good blue, and the hydrogen peroxide or peroxidase coloring became greener and clear, instead of deep blue. For longer exposures the reactions were still slower.

At 79° C. to 81° C. for thirty minutes the guaiac reaction was very slight; the peroxide light blue, and the color faded.

At 85° C. for five or ten minutes there was almost no reaction to gum guaiac, and the peroxide reaction was green and clear and slow; while at 86° to 87° C. for twenty and thirty minutes there was no reaction to either. Therefore the thermal destruction point is 86° C. for long exposures and 87° C. for shorter times, that of the oxidase being probably a few degrees lower than that for the peroxidase; the third extractions were a trifle more sensitive than the original one.

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* Green, J. R. The soluble ferments and fermentation. 1899.

† Loew, O. Physiological studies of Connecticut leaf tobacco, 31. (U. S. Dept. Agriculture Rep. No. 65.) 1900.

These conclusions are drawn from over fifty experiments made under the same conditions with the various solutions, each solution being tried at least twice, generally more often, at each degree of temperature between the extremes indicated.

A little different temperature test was made by holding branches with the leaves on in water at 70°, 80°, 90° and 100° C. for five, ten and fifteen minutes, then mashing the leaves and precipitating with alcohol and testing as usual. In this way the enzyme was not destroyed until the branch had been at 90° C. for fifteen minutes, and even then there was a little reaction with hydrogen peroxide. At the boiling point of water the oxidase was destroyed by a five-minute exposure, the peroxidase by a ten-minute exposure. Even this high temperature for less than five minutes does not quite destroy the enzyme, and this fact was noticed with a solution which gave reactions when brought once to the boiling point, and ceased only when boiled several minutes. The leaves on the branches when exposed to the lower degrees for short periods turned dark or black on drying, while the boiled ones remained green.

These temperature results correspond fairly well with those given for the maple leaf by Woods.* He found that in the plant tissues both the oxidase and the peroxidase will stand somewhat higher temperature than in aqueous or alcoholic solutions. The oxidase of a hard maple leaf is usually destroyed by heating five minutes at 70° C. in an aqueous solution; the peroxidase requires five minutes at 80° C. In the leaf, however, it took fifteen minutes at 85° C. to destroy them, while both were destroyed in a 50 per cent. alcoholic solution in three minutes at 70°–72° C. The enzymes of other leaves behaved in a similar manner. The tobacco oxidase is destroyed between 65° and 66° C. and the peroxidase between 87° and 88° C. On this subject Loew † says: "The temperatures thus far observed which render enzymes inactive are not constant under all conditions; the acidity of the plant juice, the degree of dilution, the duration of the heating, and the presence of certain salts have a modifying influence on the height of the temperature at which the change to the inactive modification takes place." He then gives destruction temperatures for several oxidases in various plants, which vary from 60° to 95° C.

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* Woods, A. F. The destruction of chlorophyll by oxidizing enzymes. Centralbl. Bakteriol. Parasit. Abt. 2. **5**: 745–754. 1899.

† Loew, O. Physiological studies of Connecticut leaf tobacco, 21. 1900.

*Citric acid tests.*—Ten cubic centimeters of the enzyme solution were put in tubes, then by means of a burette 1 cc., .5 cc., and .2 cc. of citric acid of different strengths were added, the tubes set aside for an hour and then tested with the solution of gum guaiac and hydrogen peroxide. Citric acid (normal) was used to begin with, then one fourth normal, one eighth normal and one sixteenth normal, each one tried in three tubes with the three different amounts of acid. The normal solution gave no result at all, except a heavy brown precipitate.

The quarter normal, when only two tenths cubic centimeters were added, gave a weak guaiac reaction and a gray-blue peroxide coloration.

With one half a cubic centimeter of the one eighth normal solution there was a fair reaction with both, but not normal in color, the good colors being reached only with .2 cc. of the one sixteenth normal solution. In all but the last there was a precipitate ; in the stronger solutions this was brown, in the weaker blue, and in most the color faded. No definite conclusions could be drawn from these results.

*Sodium hydroxide tests.* —These were made in the same way as those with citric acid : a one quarter normal solution was the strongest used (ten grams in one litre of water), the weaker ones being one quarter, one eight and one sixteenth of that. Results follow :

One quarter normal — no reaction with either guaiac or hydrogen peroxide, a clear yellow liquid.

| $\frac{1}{16}$ normal. | $\frac{1}{32}$ normal. | $\frac{1}{64}$ normal. |
|---|---|---|
| (10 cc. $\frac{1}{4}$ normal and 30 cc. $H_2O$.) | (5 cc. $\frac{1}{4}$ normal and 35 cc. $H_2O$.) | ($2\frac{1}{2}$ cc. $\frac{1}{4}$ normal and $37\frac{1}{2}$ cc. $H_2O$.) |
| .5 cc. in a few cases gave a slight reaction to guaiac, none to $H_2O_2$. .2 cc. and less gave generally a guaiac reaction, occasionally one with $H_2O_2$. | Most of these experiments gave a guaiac reaction, but none with $H_2O_2$ until only .2 cc. was used. | All gave guaiac reaction, though greenish instead of blue, and with .5 and .2 cc. there was a reaction to $H_2O_2$, though green instead of blue. |

The sodium hydroxide solution gave a yellow color to the enzyme solutions which was noticeable even in the weaker ones, making the color reactions greenish instead of blue. There was no precipitate in any and the color faded in most of them. In these results it is seen that it is the oxidase which is more resistant, while in the thermal tests it was the peroxidase. Loew * states that in the majority of cases the oxidase will prove the more energetic of the two but it succumbs much more quickly to noxious influences, as alcohol or heat.

A few experiments were tried with both citric acid and sodium hydroxide, using for each .5 cc. of $\frac{1}{8}$ normal solution (where a slight though decided reaction to both guaiac and hydrogen peroxide was observed), and then heating the tubes. It was found that the thermal destruction point was thus reduced from 86° to 79° C.

### MISCELLANEOUS EXPERIMENTS

1. The experiments given above seemed to prove that there were two oxidases and it was thought probable that catalase was also present, but although two attempts were made to prove this neither was successful. Hydrogen peroxide was poured on freshly chopped leaves and though bubbles came off, when collected they proved to be only air, not oxygen. However, when hydrogen peroxide was dropped on freshly made hand-sections of the stem many air-bubbles came off, especially from the wood and xylem, which action may have been due in part to catalase, as this property of developing oxygen by decomposition of hydrogen peroxide is one which Loew † proved to belong to catalase.

2. Tubes with enzyme solution were exposed to sunlight and strong light for several hours, together with controls wrapped in black paper, so that all were at the same temperature, but all gave perfectly normal reactions.

3. A solution of hydroquinone was made up (one gram in 100 cubic centimeters of distilled water), and equal amounts of this and the enzyme solutions, and one tube of distilled water and hydroquinone were put aside in the dark, for twenty four hours. As no result was apparent for this period they were left for a

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* Loew, O. Curing and fermentation of cigar leaf tobacco, 30. 1899.

† Loew, O. Physiological studies of Connecticut leaf tobacco, 23. 1900 — Loew, O. Catalase. 1901.

week; then the solutions had all become a rich, red, clear sherry color, that with distilled water also having a reddish tinge.

4. One drop of twenty per cent. ferric chloride turned a solution of freshly chopped leaves in water a deep black, showing the presence of tannin, but none could be detected in the alcoholic extracts.

5. The various extracts seemed to be quite neutral, that is, they produced no change in either blue or pink litmus paper, even when kept some days so that there was a decided musty or cider smell. A few drops of chloroform do not affect the action of the enzymes and keep the solutions from growing musty.

In the field those branches which had turned black were in general injured in some way, by being stepped on, blown over or sometimes some insect eggs were found in the hollow stems of older branches. These are some suggestions as to the possible cause for the starting of the action of the enzymes.

In the plants which are described in Woods' interesting paper he enzymes show much greater activity in or near the cells which are injured in a leaf than in the normal green cells. He suggests that the insects which cause punctures may "inject substances possibly related to saliva into the wounds which they produce, and it may be the irration caused by these substances which is responsible for the increase of oxidase and peroxidase." He also states that these enzymes rapidly destroy chlorophyl and that "under certain conditions not yet well understood" they "either become active or else are produced in abnormally large quantities, causing variegations and various diseases." *

"The oxidizing enzymes are evidently contained in the plasmatic living part of the cell, and not in the cell sap which fills the vacuoles. On the other hand, the matters easily oxidized by them and representing products of metabolism or by-products of certain synthetical operations are mostly contained in the cell sap. These matters, often of a chromogenic character, are, of course, produced in the plasmatic parts, but secreted rapidly into the cell sap. When the cells die the soluble substances locally separated in the cytoplasm and the cell sap intimately mix, since the coagulating protoplasm, becoming easily permeable, can no

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* Woods, *l. c.* 754.

longer retain any soluble matters—as, for example, oxidase. Thus after death the direct action of the oxidase on the oxidizable matter of the cell sap sets in with a production of colored bodies, as is the case in the tobacco leaves. This action can also be noticed very well in the juice pressed out from the fresh leaves, since this juice, containing cell sap as well as soluble matters previously contained in the protoplasm, turns brown rapidly on coming into contact with air. The darkening of the juices of potatoes, beets, and other plants depends upon the same principle." *

It might be concluded from these two extracts that though present in the whole plant of *Baptisia tinctoria*, the enzymes have become particularly active in the blackened parts, and that these black parts have become susceptible to the enzyme from having lost their vitality from some cause. The black leaves do not seem dead, and always contain plenty of chlorophyl. Of course the blackness is the product of oxidation, but my observations would suggest a possibility that there is a pigment as well, which gives the extreme dark color, so much darker than the same product in potatoes and beets and tobacco, and so different from the white and green of variegated leaves.

## SUMMARY

The blackening of the leaves of *Baptisia tinctoria* is due to oxidizing enzymes.

There are at least two enzymes, an oxidase which gives an opalescent blue with gum guaiac solution and is destroyed by heat at about 83°–84° C., and a peroxidase which gives a deep blue with hydrogen peroxide and which has a thermal destruction point of 86°–87° C.

Both enzymes can be destroyed with dilute solutions of citric acid and sodium hydroxide.

I wish to express my gratitude to Dr. D. T. MacDougal and Dr. W. J. Gies of the New York Botanical Garden, for their kind assistance and advice in revising this paper.

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* Loew, O. Physiological studies of Connecticut leaf tobacco, 40, 41. 1900.